

PRO-INFLAMMATORY FIBRINOPEPTIDE

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REFERENCE TO SEQUENCE LISTING

115
A1
1

[0001] The present application incorporates by reference SEQ ID NO: 1 and SEQ ID NO: 2 provided herewith on a diskette, created on August 8, 2001 and containing 5,701 bytes. The information recorded on the diskette is identical to the written sequence listing provided herein.

FIELD AND HISTORICAL BACKGROUND OF THE INVENTION

[0002] The present invention is directed to the treatment of rheumatoid arthritis (RA), and more particularly to a synthetic peptide sequence that has been discovered to induce pro-inflammatory effects in human synovial cells.

In the clotting of blood, a large protein dissolved in the plasma, known as "fibrinogen", is cut-up by an enzyme into an insoluble protein known as "fibrin", and other smaller fragments. Fibrin polymerizes to form the tough protein clot involved in blood coagulation. In addition to blood clots, polymerized fibrin is

found in many inflamed, injured tissues, some of which are not the site of gross bleeding. Specifically, fibrin is found deposited on the inflamed joint surfaces of rheumatoid arthritis patients, in association with certain other inflammatory diseases, and with certain cancers. In rheumatoid arthritis, the amount of fibrin in the synovial membranes of the inflamed joints was found in the late 1970's to correlate with the severity of the disease, although the exact mechanism behind this relationship was not known.

[0003] Fibrin deposition, long recognized as a hallmark of acute and chronic inflammatory processes, has been localized within various inflamed tissues by histological, ultrastructural, and immunofluorescent procedures (References 1 and 2). A common theme of numerous studies is that fibrin plays an active role in the induction of inflammation (References 3-8), and demonstrate that fibrin can function beyond its classic role as a hemostatic plug or temporary matrix in response to injury. However, only recently has the direct impact of fibrin metabolism on the inflammatory process been seriously investigated, and specific roles assigned to fibrin or its products as mediators of the process (References 9-12). Although fibrin is abundantly present in many inflamed tissues rich in fibroblastic cells, no significant data on fibrin(ogen) metabolite induced gene expression by fibroblasts (FB) have been published.

[0004] Previously, research has focused on the cytokine pathways of adhesion molecule induction and adhesion molecule-related mechanisms thought to be important in connective tissue diseases, such as Rheumatoid Arthritis (RA) and Scleroderma (SD). In these diseases, evidence for altered

fibrinolysis has been demonstrated, for example, in plasma and synovial fluids in RA (References 13-14). It has long been recognized that in most inflamed joints, the coagulation system is activated leading to the local generation of fibrin (Reference 15), and it has been hypothesized that the local fibrin deposition in arthritic joints could promote inflammation and destruction (Reference 16). Indeed, animal studies in which fibrin is implanted locally within joints induces a reaction that resembles human RA (Reference 28). Currently, studies are attempting to correlate associations between synovial fibrinolysis and levels of joint destruction in RA (Reference 17) and fibrinolysis with the severity of the disease state in SD (Reference 18), two diseases in which resident fibroblasts (FB) appear to play crucial roles. However, direct effects of fibrin on FB have not been previously examined with regard to the generation/maintenance of inflammation within connective tissue. Because of the universal nature of fibrin deposition in injured and inflamed tissue, determining the mediators, mechanisms and consequences of fibrin-induced activation of FB is important in not only understanding the pathways, but developing important new insights into the regulation of inflammation in connective tissue. Previous research pursued this avenue of investigation and it was found that fibrin clots could induce adhesion molecule expression in human synovial fibroblasts.

[0005] The treatment of RA has two clinical objectives: symptomatic reduction of pain and inflammation and prevention of joint damage. Scientists are experimenting with new drugs and biological agents that selectively block

certain immune system activities associated with inflammation. Recent studies suggest that these represent promising approaches to treatment.

[0006] Current therapy for RA consists of non-steroidal anti-inflammatory drugs (NSAID's), and as the disease progresses, oral steroids. Finally, disease modifying anti-rheumatic drugs (DMARD's) are added to the course of therapy.

[0007] It should be noted that no currently available agent truly improves the outcome of RA. DMARDs, such as methotrexate and sulfasalazine interfere with the inflammatory process but do not reverse or halt the progression of RA over long-term therapy. Moreover, while these agents initially work for most patients, both DMARDs and NSAIDs can cause serious side effects. And, while initial clinical response to DMARDs occurs in about 70-80% of patients, the effectiveness diminishes to approximately 20% of patients over time. It is estimated that 10% of the 2.5 million RA patients in the United States do not respond to current therapies.

[0008] Development of novel treatments for RA has been facilitated by two important factors: (1) progress in the understanding of the immunopathogenesis of RA, and (2) developments in biotechnology.

OBJECTS AND SUMMARY OF THE INVENTION

[0009] The principal object of the present invention is to provide a peptide (or fibrinopeptide or tetrapeptide) that has pro-inflammatory effects on

human synovial cells and little to no effects on human foreskin fibroblasts. The peptide bears homology with a natural sequence found in the human fibrin molecule formed during fibrinogenesis, and is a potent inducer of several pro-inflammatory mediators in human synovial cells.

[0010] The peptide of the invention is believed to play a role in inflammatory lung diseases in which fibrin deposition is also known to occur. My research has proven that human lung fibroblastic cells are also responsive to the fibrinopeptide, and under its influence, will express selective pro-inflammatory molecules on the cell surface and will secrete pro-inflammatory mediators, such as cytokines and chemical attractants for immune cells.

[0011] Another object of the present invention is to provide a peptide which could be used as a valuable tool in exploring the efficacy of clinical treatments in RA and other diseases in which fibrin is suspected of playing an inflammatory role. For instance, if one wishes to design new anti-inflammatory compounds to address or subdue this new pathway of inflammation, or to evaluate the effectiveness of existing compounds or treatments on fibrin-induced inflammation, the four amino acid peptide of the invention is a cleaner system of cell activation than using the entire fibrin molecule. In in vivo animal studies (required testing), intact fibrin would be expected to undergo additional cleavage and folding reactions within tissues and many of the resulting fragments are known to have myriad effects on different cell types which would make interpretation of results difficult. The use of the isolated fibrinopeptide of the invention eliminates this difficulty.

[0012] Yet another object of the present invention is to provide a peptide which has the significant advantage in that it can be used to identify, isolate and clone the cell receptor to which it binds. This would likely lead to significant commercial value.

[0013] An additional object of the present invention is to provide an analog of the peptide which is ineffective in inducing, or effective in causing a loss of, pro-inflammatory effects. This is believed to be particularly useful in the treatment or prevention of inflammation of a synovial joint, and related disorders, such as rheumatoid arthritis.

[0014] Yet an additional object of the present invention is to provide all analogs, mutants, fragments, derivatives, functional homologs and other variants of the peptide of the invention.

[0015] In summary, the present invention is based on the discovery that a synthetic four amino acid peptide has pro-inflammatory effects on synovial cells. The peptide of the invention has an amino acid sequence of Glycine-Proline-Arginine-Proline. An amide analog of the peptide has been found to be ineffective in inducing, or effective in causing a loss of, pro-inflammatory effects.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] The above and other objects, novel features and advantages of the present invention will become apparent from the following detailed description of the invention, as illustrated in the drawings, in which:

[0017] Figure 1 (A-C) illustrates an amino acid sequence of human fibrinogen A α chain set forth in SEQ ID NO:1;

[0018] Figure 2 illustrates cleavage of fibrinopeptide A by thrombin;

[0019] Figure 3 illustrates the natural sequence of amino acids 17 through 20 of the A α chain of fibrinogen;

[0020] Figure 4 illustrates the amino acid sequence of the peptide of the invention set forth in SEQ ID NO: 2; and

[0021] Figure 5 is a two-dimensional diagram based on X-ray structure illustrating hydrogen bonds and electrostatic interactions between the G-P-R-P knob and the γ -chain hole.

DETAILED DESCRIPTION OF THE INVENTION

[0022] The amino acid sequence of the pro-inflammatory peptide of the invention is Gly-Pro-Arg-Pro (GPRP). This sequence is related to amino acids 17 through 20 of the A/ α chains of fibrinogen (Figure 1). Amino acids 1 through 16 of the A/ α chains are cleaved-off by thrombin or thrombin-like proteases in vivo and this now separated peptide is known as Fibrinopeptide A. Cleavage of Fibrinopeptide A by thrombin is the initial cleavage in the formation of fibrin clots from its precursor, fibrinogen (see Fig.2). This cleavage exposes new amino-termini of the A/ α chains (beginning with amino acid number 17) with the leading sequence Gly-Pro-Arg. These newly exposed sequences, which are centrally located in a spatial model of fibrinogen have been referred to as

"knobs", and are able to bind to complementary "holes" in the gamma chains of the same or different fibrinogen molecules and thus facilitate the crosslinking of fibrin(ogen) into a meshwork, or clot (see Fig.5). I believe that there exists another receptor-like "hole" on the surface of human synovial cells, through which the "knobs" can mediate pro-inflammatory effects.

[0023] Synthetic peptides modeled after these "knobs" are able to inhibit fibrin polymerization by covering up the "holes" on the gamma chains and are commercially available. The natural sequence of the new amino terminus following Fibrinopeptide A cleavage is Gly-Pro-Arg-Val (GPRV) and this peptide functions well as a polymerization inhibitor (Reference 25). It was found that the modified peptide Gly-Pro-Arg-Pro was an even more potent inhibitor of polymerization, although the reason why is not understood and is hypothesized to be due to steric constraints (rigidity) imposed by the terminal proline. Many studies of fibrinogen polymerization make use of such synthetic peptides. I first used a commercial GPRP to inhibit fibrin polymerization in studies of fibrin co-culture with endothelial cells and have published our results (Reference 19). I found GPRP to be a good inhibitor of fibrin polymerization yet had no direct effects itself on endothelial cells. I also found GPRP to have low to no effects on other cell types, such as foreskin fibroblasts.

[0024] I derived several primary cell lines from the synovium of RA and osteoarthritis (OA) patients. I wanted to examine the effects, if any, of polymerized fibrin on synovial fibroblasts in vitro, to mimic the environment found in vivo in a rheumatoid joint. I was surprised to find that the inclusion of GPRP

along with fibrinogen (to inhibit polymerization) did not abrogate my initial findings of increased ICAM-1 (Intercellular Adhesion Molecule-1) expression (caused by exposure to fibrin clots) by the synovial FB. ICAM-1 is considered to be such a key component in the inflammation of the rheumatoid joint, that RA therapeutic trials have been based on the inactivation of ICAM-1 alone. Indeed, I found that the GPRP peptide alone was able to induce ICAM-1 expression by the synovial fibroblasts as measured both by ELISA and flow cytometry. By flow cytometry, GPRP peptide increased ICAM-1 expression in two ways: 1) increasing the percentage of cells expressing ICAM-1, and 2) increasing the amount of surface ICAM-1 per cell. I conducted experiments with more than seven different RA fibroblast lines and obtained consistently increased expression of ICAM-1 induced by GPRP with all lines. My experience is that GPRP is able to reliably induce the adhesion molecule ICAM-1 about as well as any cytokine. The effects of GPRP on ICAM-1 induction are dose-dependent. A significant induction at 1.0, 0.5, and 0.1 mg/ml was observed. Induction at 0.01 mg/ml was found not to be significant.

[0025] Table 1 illustrates an experiment using GPRP to increase ICAM-1 expression on different RA synovial lines assayed by flow cytometry, and compares those results with the failure of GPRP to induce ICAM-1 on human foreskin fibroblasts.

Table 1

FLOW CYTOMETRY ANALYSIS OF ICAM-1 EXPRESSION ON HUMAN
FIBROBLASTS

Fibroblasts were incubated with culture medium (control) or medium containing fibrin clot (0.6 mg/ml) or the tetrapeptide of the invention, GPRP (1 mg/ml). Fibroblasts were washed and incubated with saturating concentrations of anti-ICAM-1 antibody followed by staining with FITC-conjugated goat anti-mouse IgG. Positive regions were set to exclude 97% of non-specifically stained cells, determined using an irrelevant primary antibody.

Cell Line	%positive	MFC
RA line 9 control	43.4	1.09
RA line 9 + fibrin	68.5	2.17
RA line 9 + GPRP	80.1	3.62
RA line 4 control	45.5	1.47
RA line 4 + fibrin	72.0	1.53
RA line 4 + GPRP	87.0	2.73
RA line 5 control	33.3	1.09
RA line 5 + GPRP	74.5	2.00
RA line 6 control	86.3	2.41
RA line 6 + GPRP	95.0	9.19
Foreskin fibroblasts control	85.6	1.89
Foreskin fibroblasts + GPRP	86.7	2.26

[0026] Table 2 illustrates an experiment using GPRP to increase ICAM-1 in different RA synovial lines assayed by ELISA and compares those results with the failure of GPRP to induce ICAM-1 on human foreskin fibroblasts.

TABLE 2
ICAM-1 EXPRESSION BY ELISA

Synovial or foreskin fibroblasts were incubated overnight with either control medium, fibrin clots (0.6 mg/ml), or GPRP (1 mg/ml). Cell-ELISA was performed the following morning for ICAM-1 expression on the cell surface. Optical Density was read at 492 nm. Results are expressed as mean \pm standard deviation for triplicate determinations.

Cell Line	O.D. 492
RA line 9 control	0.44 \pm .11
RA line 9 + fibrin	1.15 \pm .04
RA line 9 + GPRP	0.98 \pm .15
RA line 4 control	0.48 \pm .05
RA line 4 + fibrin	1.19 \pm .14
RA line 4 + GPRP	1.04 \pm .02
RA line 5 control	0.59 \pm .02
RA line 5 + fibrin	1.16 \pm .06
RA line 5 + GPRP	1.10 \pm .03
Foreskin fibroblasts control	0.46 \pm .06
Foreskin fibroblasts + fibrin	0.59 \pm .13
Foreskin fibroblasts + GPRP	0.55 \pm .05
Foreskin fibroblasts + interferon γ (200 U/ml) (positive control for foreskin fibroblasts)	1.57 \pm .13

As can be observed, the ability of GPRP to induce ICAM-1 on synovial fibroblasts is as good as whole fibrin clot.

[0027] Table 3 shows GPRP induction of IL-6 (measured by ELISA).

Table 4 shows a dose-dependent increase in ICAM-1 expression on synovial FB by GPRP.

TABLE 3

GPRP INDUCTION OF IL-6 - MEASURED BY ELISA

Synovial or foreskin fibroblasts were incubated overnight with and without GPRP. An IL-6 ELISA was performed the following day on the cell supernatants.

Cell Line	Control pg/ml	GPRP pg/ml	INCREASE
RA #1	1600	2400	1.50X
RA #11	275	1700	6.18X
RA #16	3000	11100	3.70X
RA #18	490	940	1.90X
Foreskin Fibroblasts	255	215	—

TABLE 4

**GPRP INCREASES ICAM-1 ON SYNOVIAL FIBROBLASTS IN A DOSE
DEPENDENT MANNER**

Synovial and Foreskin fibroblasts (FF) were incubated overnight with varying concentrations of GPRP. Cell ELISAS were performed the following day for ICAM-1 expression on the cell surface.

	RA#11 OD 492	RA#12 OD 492	FF- #1 OD 492	FF- #2 OD 492
Control	.25 ± .02	0.53 ± .05	0.95 ± .01	.72 ± .04
GPRP 1.00 mg/ml	.59 ± .05	1.42 ± .08	1.02 ± .01	.78 ± .04
0.30	.46 ± .05	1.09 ± .06	0.99 ± .01	.72 ± .03
0.10	.37 ± 0	0.76 ± .03	0.92 ± .02	.72 ± .02
.030	.31 ± .02	0.62 ± .03	0.94 ± .05	.70 ± .02
.010	.24 ± .05	0.57 ± .02	0.93 ± .03	.71 ± .02
.003	.25 ± .03	0.53 ± .01	0.95 ± .06	.71 ± .04
.001	.24 ± .05	0.51 ± .01	0.92 ± .01	.69 ± .02

[0028] I also assayed several lines for V-CAM-1 expression, and found that about 50% of the lines tested upregulated V-CAM-1 expression in response to the peptide. I also found that VCAM-1 induction is not as robust as ICAM-1.

[0029] I have confirmed an upregulation of functional adhesion molecules by GPRP using a human in vitro T cell adhesion assay (see Table 5 below).

TABLE 5

GPRP INDUCES T CELL ADHESION

Synovial (RA and OA derived) or foreskin fibroblast cells were incubated with culture medium (control) or medium containing IL-1 beta (1ng/ml, an inducer of ICAM-1) or GPRP (1.0 and 0.1 mg/ml) overnight. Cells were washed and 100 ul of fresh purified human T cells were added for one hour and non-adherent T cells were removed by washing. Adherent T cells (known to be adherent predominantly via ICAM-1 interaction with T-cell LFA-1) were counted after the cultures had been air dried and stained with 1% methylene blue. Results shown are T cell numbers \pm standard deviations for triplicate wells counted using an ocular grid.

Cell Line	Control	IL-1	GPRP(1mg/ml)	GPRP (0.1 mg/ml)
RA line 7	80 \pm 8	116 \pm 14	153 \pm 54	184 \pm 58
RA line 8	29 \pm 2	58 \pm 21	97 \pm 10	99 \pm 18
OA line 15	35 \pm 6	66 \pm 19	113 \pm 44	92 \pm 27
Foreskin line	47 \pm 10	94 \pm 16	54 \pm 11	51 \pm 3

[0030] One consideration in evaluating the effects of any substance on my collection of RA synovial cell lines, is that the synovial tissues I used were collected sequentially as the patient surgeries were scheduled. Therefore, I had no knowledge of the patients' disease history, or what medications they might have been taking or for how long.

[0031] Another effect of the peptide of the invention on synovial FB, is the induction of the chemotactic cytokines IL-8 and GRO-alpha, each of which is intimately involved in the recruitment of lymphocytes into inflamed tissues. I used commercial ELISA kits to assay cell culture supernatants of RA fibroblasts that were cultured for both 24 and 48 hours with or without peptide. RA Line 1 increased its production of IL-8 12.6-fold at twenty-four hours and fifteen-fold at forty-eight hours. RA Line 2 behaved similarly with ten-fold increases at both twenty-four and forty-eight hours. For comparison, monocytes, considered to be excessive IL-8 secreters, increase IL-8 production approximately three-fold when stimulated with phytohemagglutinin (PHA). It is believed that a technical error in the absorbance readings only of our GRO-alpha assay may have resulted in a very significant underestimation of the amount of GRO-alpha secreted in response to peptide. However, in a visual observation of developed ELISA plates, the fold increase was at least as much as was seen for IL-8 and probably is greater. Using foreskin FB as control cells, I accurately measured only a 1.2 fold increase in GRO-alpha secretion in response to the peptide both at twenty-four and forty-eight hours. I believe that not only does GPRP participate in the chemotactic recruitment of lymphocytes via induction of IL-8 and GRO-alpha, but it gives the lymphocytes a place to "park" once they arrive via the simultaneous induction of adhesion molecules such as ICAM-1 and VCAM-1.

[0032] Table 6 presents our ELISA results for the chemotactic cytokines IL-8 and GRO-alpha.

TABLE 6

**GPRP INDUCTION OF CHEMOKINE SECRETION FROM HUMAN SYNOVIAL
FIBROBLASTS**

Synovial or foreskin fibroblasts were incubated with control medium or medium containing 1 mg/ml GPRP for 24 and 48 hours. Cell culture supernatants were tested by commercial ELISA kits for the chemokines IL-8 and GRO- α (R & D Systems, Minneapolis, MN). All samples were additionally tested for Interferon γ secretion. All supernatants were negative for Interferon γ secretion.

IL-8 (pg/ml)			
Cell Line	Control	24 hr	48 hr
RA line 1	1260	16,000	17,500
RA line 1	1140		
RA line 4	900	9,000	6,800
RA line 4	680		
Foreskin fibroblasts	400	1,840	1,200
Foreskin fibroblasts	260		

GRO- α		
Cell Line	GRO- α pg/ml	Fold Increase
RA line 7 Control	55	145X
Fibrinogen	8000	
RA line 8 Control	400	21X
GPRP	8500	
Foreskin fibroblasts Control	380	1.2X
GPRP	470	

[0033] In another experiment using RT-PCR, it was found that synovial cells exposed to GPRP had significantly more IL-6 gene expression than controls. IL-6 has been demonstrated to be both a chemoattractant and pro-inflammatory cytokine in RA.

[0034] The GPRP peptide of the invention is believed to induce gene expression of ICAM-1 through its stimulation of the intracellular signal NF-kappaB which regulates the intensity of gene transcription. NF-kappaB activation is already well known to affect a broad array of immediate-early gene products, such as TNF, interleukins, chemokines and colony stimulating factors; genes that are tightly regulated during inflammation and wound healing. Inclusion of the specific inhibitor of NF-kappaB, pyrrolidinedithio-carbamate (PDTC), stopped the entire increase in ICAM-1 expression induced not only by Interleukin-1 (as expected), but also by the GPRP peptide. PDTC is an anti-oxidant that prevents NF-kappaB activation and its translocation to the nucleus (Reference 26). As NF-kappaB is also known to be involved in IL-8 transcription, it is expected that PDTC would also inhibit the IL-8 secretion induced by GPRP.

[0035] Table 7 shows the inhibitory results of PTDC on the ability of GPAP to induce ICAM-1.

TABLE 7

PDTC INHIBITS IL-1 AND GPRP INDUCTION OF ICAM-1 IN HUMAN
SYNOVIAL CELLS

Cells were pre-incubated with PDTC for 1.5 hours before the addition of IL-1 (positive control) or GPRP overnight. ELISA assay for ICAM-1 was performed the following morning. Results are presented as O.D.492 \pm standard deviation for triplicate determinations.

	RA line 6	RA line 5	OA line 8	OA line 2
Control	0.66 \pm .03	0.85 \pm .02	0.53 \pm .03	0.69 \pm .02
IL-1	1.80 \pm .04	1.88 \pm .05	1.51 \pm .05	1.60 \pm .03
IL-1 + PDTC	0.70 \pm .06	0.85 \pm .07	0.60 \pm .02	0.57 \pm .01
GPRP	1.67 \pm .06	1.83 \pm .08	1.30 \pm .09	1.66 \pm .05
GPRP + PDTC	0.61 \pm .02	0.75 \pm .09	0.60 \pm .02	0.65 \pm .02
PDTC alone	0.61 \pm .02	0.75 \pm .06	0.55 \pm .04	0.65 \pm .03

[0036] Most modifications of the GPRP sequence (amino acid substitutions) are not effective polymerization inhibitors. For example, changing Gly1 to anything else destroys its inhibitory action as there appears to be no extra "room" for a side chain in the gamma chain "hole" into which it must fit. However, as noted below, amino acid Pro4 functions differently. Actually, Pro4

is not essential for activity, as Gly-Pro-Arg alone can inhibit fibrinogen polymerization, although inhibition is much more effective when Pro4 is included.

[0037] Changing the carboxyl group at the end of Pro4 to an amide group (OH to NH₂) has been found to be approximately three and one-half times more effective at inhibiting polymerization as the original H-Gly-Pro-Arg-Pro-OH (Reference 20). Interestingly, when I substituted the amide analog of GPRP, it was totally ineffective at inducing ICAM-1 in synovial fibroblasts. Although it is not yet shown that the amide form of GPRP still binds to the synovial cell receptor, it is unlikely that this change in the number four position would compromise binding ability. Further research is contemplated in using the amide analog of the peptide of the invention to determine if other pro-inflammatory responses of the synovial FB are also prevented.

[0038] Table 8 contains data comparing the activities of GPRP-OH and GPRP-NH₂. This Table also contains data showing that synovial cell incubation with other, coagulation-related peptides (Fibrinopeptides A and B, and an amino acid sequence which prevents platelet aggregation by binding to the fibrinogen receptor) have no effect on ICAM-1 induction.

TABLE 8

AMIDE FORM OF GPRP IS INEFFECTIVE AS AN INDUCER OF ICAM-1 ON
HUMAN SYNOVIAL FIBROBLASTS.

Synovial cells from patients with rheumatoid arthritis (RA), osteoarthritis (OA) or foreskin fibroblasts were incubated overnight with culture medium (control), or culture medium containing one of the following: 1) IL-1 (0.5 ng/ml), 2) GPRP (1 mg/ml), 3) GPRP-amide (1mg/ml), 4) fibrinogen receptor peptide N-acetyl-Pen-Arg-Gly-Asp-Cys, (0.1 mg/ml, related control peptide), or 5) Fibrinopeptides A and B, (FPA, FPB, 10^{-4} M each). ICAM-1 was determined by ELISA and results are presented as O.D. 492 \pm standard deviation of triplicate determinations.

	RA line 1	RA line 2	OA line	Foreskin line
Control	0.64 \pm .02	0.43 \pm .003	0.47 \pm .04	0.74 \pm .04
IL-1 (pos control)	1.74 \pm .08	1.18 \pm .03	1.36 \pm .03	1.77 \pm .13
GPRP	1.38 \pm .16	0.98 \pm .03	0.85 \pm .08	0.79 \pm .02
GPRP-amide	0.65 \pm .02	0.49 \pm .02	0.44 \pm .01	0.80 \pm .01
Fib.Recept. Pept.	0.56 \pm .02	0.42 \pm .01	0.38 \pm .02	0.73 \pm .08
FPA	0.61 \pm .05	0.43 \pm .03	0.31 \pm .005	0.69 \pm .02
FPB	0.67 \pm .04	0.47 \pm .02	0.41 \pm .04	0.80 \pm .02

[0039] Table 9 illustrates an analysis of the binding of biotinylated amino acid sequence 17 – 28 of human fibrinogen A α Chain (SAC[KBtn]) to RA synovial fibroblasts by Flow Cytometry. The biotinylated control peptide (KREE) represents corresponding sequences derived from the B/beta chain of fibrinogen following removal of Fibrinopeptide B.

TABLE 9

GPRVVERHQSAC[KBtn] = SAC [KBtn]
GHRPLDKKREE[KBtn] = KREE[KBtn] = control peptide

RA #16		% Cells Positive	Mean Fluorescent Channel
Avidin-FITC		3.6	13.71
SAC [KBtn]	200 ug	80.0	125.1
SAC [KBtn]	100 ug	86.3	257.9
KREE [KBtn]	100 ug	10.6	20.37

[0040] Table 10 illustrates Flow Cytometry Analysis of SAC Competition Assay.

TABLE 10

Synovial cells were incubated with unlabeled Fgn(2.5mg), SAC(2.5mg) or GPRP(2.5mg) for 3 hours followed by labeled SAC[KBtn] for 1 hour

	Mean
AVIDIN-FITC	20.16
SAC[KBtn], Avidin-FITC	51.20
Fgn: SAC[KBtn], Avidin-FITC	21.63
SAC: SAC[KBtn], Avidin-FITC	36.30
GPRP: SAC[KBtn], Avidin-FITC	33.58

[0041] These data suggest that unlabeled fibrin, unlabeled GPRVVERHQSAC (SAC) and unlabeled GPRP can compete with biotin-labeled SAC for binding to human synovial fibroblasts. This competition further suggests that the labeled peptide competitively binds to a specific cell surface receptor of synovial cells. Thus, labeled peptide could facilitate the isolation, identification and characterization of its receptor, through standard immunological techniques such as immunohistochemistry, flow cytometry and immunoprecipitation.

[0042] The data collected thus far leads me to believe that there exists on the surface of human synovial cells, a receptor that can be stimulated when

fibrinogenesis is occurring in the synovial joint. The ligand for this receptor is created when newly exposed amino acid sequences become available due to Fibrinopeptide A release from the precursor fibrinogen. This newly exposed "knob" has two options: 1) it can bind to fibrinogen gamma chains thereby promoting cross-links and the formation of fibrin clots, or 2) given the right environment (synovial cells bearing the correct receptor) can also act as ligand for these cellular receptors. Binding of the ligand stimulates intracellular signaling mechanisms some of which require NF-kappa B and results in the simultaneous expression and production of pro-inflammatory mediators, such as an array of adhesion molecules and chemotactic cytokines.

[0043] Further research is contemplated to determine what further activities synovial FB might produce under the influence of this ligand (IL-1, chemoattractant IL-16, MCP-1, prostaglandin production, matrix metalloprotease production, and collagenase-1 and cathepsin production, for example). It is believed that in vivo, fibrin-induced activation of synovial FB results in the induction of a phenotypic "Pro-Inflammatory FB" leading to the recruitment, activation, attachment and retention of lymphocytes, all of which occur to a tremendous degree in the chronically inflamed rheumatoid joint. It is well known that the recruitment and attachment of lymphocytes to synovial FB results in further amplification of inflammation. Most recent therapies directed at biologically alleviating inflammation in the rheumatoid joint have focused on inhibiting one or another facets of the inflammatory response, i.e., using anti-

ICAM-1 monoclonals, monoclonal antibodies or engineered proteins directed against TNF, etc., and are very costly.

[0044] A recent review by Lorenz, et. al (Reference 27) discusses the advantages and disadvantages of current and emerging RA therapies and describes further efforts to reduce inflammation via antibodies to specific cytokines. It is becoming apparent that studies targeting a single biological entity at a time are being replaced by studies in which they are combined with more traditional anti-inflammatory medications. Also contained in this review is a discussion of emerging results for targeting the membrane urokinase-type plasminogen activator of human synoviocytes. My findings could advance such approaches. A double blind clinical trial of stanozolol (enhances both systemic and intra-articular fibrinolytic activity) in RA patients (Reference 29) resulted in clinical benefit, i.e., decrease in erythrocyte sedimentation rate, improvement in articular index, decreased duration of morning stiffness, decrease in pain, and decreased plasma fibrinogen concentrations. It was believed that the clinical improvement likely could have been due to the induced reduction of synovial fibrin.

[0045] It is believed that once we more fully understand the effects of the peptide of the invention on synovial FB, as compared with less specialized FB found elsewhere in the body, a modification thereof, including the amide form, could be used as a binding, but non-signalling ligand, which would specifically target synovial fibroblasts, and thereby prevent the natural ligand from provoking the fibroblasts towards the pro-inflammatory phenotype. Since it

is known that procoagulant activity occurs at a heightened level in a rheumatoid joint, this would represent an important approach to subduing the inflammatory component. It may also answer the question of exactly how fibrinogenesis promotes inflammation - a fact long known to be true, but its mechanism still unknown.

[0046] I am not aware if the reported activities of all of the modified peptides that have been created in studies of fibrinogen polymerization, have any bearing on our observations. There is no evidence to suggest that the gamma chain "hole" is the same size or sequence as our putative receptor on synovial cells. Modified peptides which reportedly do not bind the gamma chain "hole" may still bind to this receptor and any peptide derivatives (previously known to inhibit fibrin polymerization or not) would have to be re-tested using our cell system.

[0047] Looking in a new direction, extravascular fibrin deposition is also frequently observed in association with neoplastic tissues in vivo. Many clinical and experimental findings (References 21-23) support the hypothesis that fibrin facilitates tumor growth and metastasis, although the mechanisms are not yet known. In a recent study of fibrin deposition in head and neck tumors (Reference 24), there was evidence of in situ thrombin activation and fibrin formation, and it was noted that the fibrin deposition was almost exclusively localized to the connective tissue compartment immediately surrounding the tumors. In 10/25 laryngeal and 4/9 hypopharyngeal cancers, characteristic fibrin accumulation was seen around tumor cell nodules, at the interface of connective

tissue and tumorous parenchyma. Tumor cell clusters were observed embedded within connective tissue "soaked with fibrin". Fibrin was not detected in the histologically normal part of tissue surrounding the squamous cell carcinomas. We look to culture connective tissue fibroblasts originating from these areas surrounding the tumors with the expectation that GPRP or other peptides like it may induce a release of factors from these specialized FB which in turn would have a "feeder", or pro-cancerous effect on the tumor cells.

[0048] The present invention also includes therapeutic or pharmaceutical compositions comprising a peptide or a peptide derivative of the invention in a form which can be combined with or in combination with a pharmaceutically acceptable carrier for any appropriate manner for administration, including, for example, oral, nasal, intravenous or intramuscular administration. Appropriate dosages, duration and frequency of administration would be determined by known factors, such as the condition of the patient, the type and severity of the disease and the method of administration. The term "carrier" includes a diluent, adjuvant, excipient, or vehicle with which the peptide is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum oil such as mineral oil, vegetable oil such as peanut oil, soybean oil, and sesame oil, animal oil, or oil of synthetic origin. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions.

[0049] Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate,

glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The therapeutic compositions, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, capsules, powders, sustained-release formulations and the like. The composition can be formulated with traditional binders and carriers such as triglycerides. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions contain a therapeutically effective amount of the therapeutic composition, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

[0050] The composition may be formulated in accordance with routine procedures as a pharmaceutical composition adapted for local injection administration to human beings. Typically, compositions for local injection administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic, such as lidocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container, such as an ampoule or sachette indicating the quantity of active agent. Where the composition is administered by injection, an

ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

[0051] The therapeutic or pharmaceutical compositions of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts, include those formed with free amino groups, such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups, such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

[0052] The present invention also provides for the modification of the peptide or peptide derivatives such that it is more stable once administered to a subject, i.e., once administered it has a longer time period of effectiveness as compared to unmodified peptide. Such modifications are well known to those of skill in the art, e.g., polyethylene glycol derivatization (PEGylation), microencapsulation, etc.

[0053] While this invention has been described as having preferred sequences, ranges, steps, materials, or designs, it is understood that it includes further modifications, variations, uses and/or adaptations thereof following in general the principle of the invention, and including such departures from the present disclosure as those come within the known or customary practice in the art to which the invention pertains, and as may be applied to the central features hereinbefore set forth, and fall within the scope of the invention and of the limits of

